The Epidermal Microbiome Within An Aggregation of Leopard Sharks (Triakis Semifasciata) Has Taxonomic Flexibility With Gene Functional Stability Across Three Time-Points

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The epidermal microbiome within an aggregation of *Triakis semifasciata* (leopard shark) has taxonomic flexibility with gene functional stability across three time-points

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Abstract

Background: The epidermis of Chondrichthyan fishes consists of dermal denticles with production of minimal but protein rich mucus that influence the attachment and biofilm development of microbes, facilitating a unique epidermal microbiome. Here, we use metagenomics to provide the taxonomic and functional characterization of the epidermal microbiome of the *Triakis semifasciata* (leopard shark) across three time-points to identify links between microbial groups and host metabolism. Our aims include 1) describing the variation of microbiome taxa over time and identify those members which are recurrent (present across all time-points, 2) investigating the relationship between the recurrent and flexible taxa (those which are not found consistently across time-points, 3) describing the functional compositions of the microbiome which may suggest links with the host metabolism; and 4) identifying whether the metabolisms are share across microbial genera or found in specific taxa.

Results: Microbial members of the microbiome showed high similarity between all individuals (average similarity: 82.74) with relative abundance of those members varying across years,

suggesting flexibility of taxa in the microbiome. One hundred and eighty-eight genera were identified as recurrent, including *Pseudomonas, Erythrobacter, Alcanivorax, Marinobacter* and *Sphingopxis* being consistently abundance across time-points, while *Limnobacter* and *Xyella* exhibited switching patterns with high relative abundance in 2013, *Sphingobium* and *Sphingomona* in 2015, and *Altermonas, Leeuwenhoekiella, Gramella* and *Maribacter* in 2017. Of the 188 genera identified as recurrent, the top 19 relative abundant genera forming three recurrent groups. The microbiome also displayed high functional similarity between individuals (average similarity: 97.65) with gene function composition being consistent across time-points.

Conclusion: These results show that while presence of microbial genera exhibit consistency across time-points, their abundances do fluctuate. Functions however remain stable across time points; thus, we suggest the leopard shark microbiomes exhibit functional redundancy. We hypothesize this may be the result of the host's epidermal attributes structuring the microbiome. In addition, we show the co-existence of many microbial genera that carry genes which may enable the microbes to use the nutrients provided by the elasmobranch's metabolism.

Keywords: Microbiome, leopard shark, *Triakis semifasciata*, shark skin, next-generation sequencing

Introduction

The epidermis of marine organisms is biologically active (i.e. not keratinised like most terrestrial organisms)(Meyer and Seegers 2012), serving as the first line of defence against environmental influences. Along with providing broad protections, the skin of elasmobranchs interacts with the microbes from the surrounding environment, forming a specific microbiome (Doane et al., 2017). The epidermal microbiome of five elasmobranchs, including thresher sharks (*Alopias vulpinus*), whale sharks (*Rhincodon typus*), leopard sharks (*Triakis semifasciata*), blacktip reef sharks (*Carcharhinus melanopterus*), and round rays (*Urobatis helleri*) confirmed that the elasmobranch epidermal microbiomes are species-specific (Doane et al. 2017; Doane et al. 2020; Pogoreutz et al. 2019). However, the selection and maintenance of the microbiome on sharks remains an outstanding question, and may be the result of several processes, including 1)

biophysical properties of the epidermis, 2) epidermal secretions of the shark, and 3) interspecific interactions between the microbes.

The biophysical properties of the chondrichthyan fish epidermis are uniquely covered with dermal denticles that improve hydro-dynamism (Lang 2020). Dermal denticles have ridges and troughs arranged in overlapping patterns that alter the hydrodynamic properties of water close to the epidermis (Sullivan and Regan 2011b; Zhang et al. 2011; Wen et al. 2015) and this biophysical property potentially affects microbial growth. In synthetic models mimicking shark skin, two bacteria, *Escherichia coli* and *Staphylococcus aureus*, showed differential growth patterns compared to a smooth surface. *E. coli* had high attachment rates to the structure surface, whereas *S. aureus* was not able to attach (Chien et al. 2020), therefore, suggesting that only specific types of microbes are able to attach to the shark skin. Further, modeling of microbial community dynamics shows that high surface structural complexity can reduce competition between microbes that result in chaotic spatial species distribution pattern on the surface (Lowery and Ursell 2019). Therefore, the high micro-structured nature of the shark skin surface, may produce similar dynamics in the microbiome. Further, the epidermis of Chondrichthyan fish is characterized by a lower level of secretory cells compared with Actinopterygian fishes (Meyer and Seeger 2012) and the product is a thin heterogeneous outer mucous layer (5–8 μm thickness; pH between 6 and 7). The mucus contains high levels of proteins, with only one protein being identified, which is lectin pentraxin (Tsutsui et al. 2014) and has moderate to high disulphide concentrations that is suspected to provide mechanical stabilization of the mucus coating (Meyer, Seegers, and Stelzer 2007).

The metabolism of Elasmobranchs may also affect the functions of the microbiomes in the form of secretions being transported by the skin mucus. Elasmobranchs rely on ketone bodies, fatty acids, carbohydrates, and amino acids for energy production (Speers-Roesch and Treberg 2010). Amino acids are ketogenic precursors, oxidative fuel, and nitrogen suppliers (Speers-Roesch and Treberg 2010). Nitrogen metabolism is also important due to the high amount of urea synthesised for osmoregulation, along with trimethylamine N-oxide (TMAO), which could also be transported to the skin surface via mucus production (Ballantyne 1997; Speers-Roesch and Treberg 2010). Sharks bioaccumulate toxins and heavy metals from lower trophic levels

(Escobar-Sanchez, Galvan-Magana, and Rosiles-Martinez 2010; Maz-Courrau et al. 2012) and have been shown to exhibit maternal off-loading to their offspring in mucus surrounding developing embryo (Lyons and Lowe, 2013). Therefore, metabolically derived compounds and harmful contaminants are being leached from elasmobranchs in their mucus and if this occurs at the skin surface may structure the skin microbiome. For instance, the microbiome signature from thresher sharks (*Alopias vulinpus*) suggests potential heavy metal concentration at the skin interface signified by high abundance of heavy metal resistance genes (Doane et al., 2017).

Interactions between the microbes on the surface and those from the surrounding water are also expected to affect the characteristics of the microbiome. The low levels of mucus secreted by the sharks and minimal biofilm development that occurred on the synthetic models of the shark skin, compared with the pronounced biofilm development on the smooth surface (Chien et al. 2020), suggests that microbial growth on the epidermis is suppressed, and the turnover of microbial communities may be high. Conversely, stability of a microbiome is important for the host's health and functioning, and rapid or unexpected variations in microbiome taxonomy leads to a state of dysbiosis, which can result in a decline in organismal health (Costello et al. 2012; Egan and Gardiner 2016). Therefore, an understanding of how the microbiome varies through time is important, but there are limited investigations of microbiome stability over time in the marine environment. A few examples include the epidermis microbiome of humpback whales (*Megaptera novaeangliae)* and Atlantic cod (*Gadus morhua*) which varied seasonally (Bierlich et al. 2018; Wilson, Danilowicz, and Meijer 2008), and similar seasonal changes occurred in the microbiome of the stony coral, *Montastrea faveolata* (Kimes et al. 2013). While the respiratory microbiome of bottlenose dolphin species (*Tursiops truncatus* and *Tursiops aduncus*) maintained taxonomic stability over two months, but individual dolphins had significantly different microbiome taxonomic structures (Lima et al. 2012). In an analysis of 8 months, captive dolphins had an individual signature of their lung microbiome and shared between 17 – 41 % of the microbiome with other individuals (Vendl et al. 2021). In contrast, 77.6 % of the microbes in the epidermal microbiome of thresher sharks (*A. vulpinus*) were shared across individual sharks (Doane et al. 2017), however, this study was only conducted at one timepoint. Therefore, whether the epidermal microbiomes of marine vertebrate hosts are temporal stable or flexible remains an outstanding question in science.

Here we analyse both the taxonomic make-up and functional capabilities of the leopard shark (*Triakis semifasciata)* epidermal microbiome over five years (three sampling time-points) using the following objectives: 1) to describe the variation of microbiome taxa over time and identify those members which are recurrent (present across time-points, 2) to investigate the relationship between the recurrent and flexible taxa (those which are not found consistently across time-points, 3) to describe the functional compositions of the microbiome which may suggest links with the host metabolism; and 4) to identify whether the metabolisms are share across microbial genera or found in specific taxa.

Materials and Methods

Epidermal Microbiome Sampling

Leopard sharks (*Triakis Semifasciata)* sharks form consistent aggregations in late summer, early fall at near-shore locations (Nosal et al. 2014; Nosal et al. 2013) along the coast of California making this species a good candidate to investigate temporal microbiome dynamics of a wild animal. Sharks in this study were sampled from La Jolla Cove, California, USA (32°51'12" N, 117°15'48" W). We targeted at least three sharks per year, and captured eight sharks in 2013, three in 2015, and seven in 2017, thus the study was conducted over a five-year period. The sharks were caught with a handline on baited barbless circle hooks and brought onboard a small skiff for processing using a scoop net; all sharks were released after processing. Microbiome samples from the sharks were collected using a microbial collection tool, which flushes the epidermis with ~250 ml of sterile seawater and dislodges the microbes. The microbial slurry is collected into the back end of the microbial tool, as we have conducted previously (Doane et al. 2017; Doane et al. 2018; Doane et al. 2020; Johri et al. 2019; Lima et al. 2020). Each epidermal microbiome sample was taken from the flank region below the dorsal fin and above the lateral line. The collected microbiome samples were filtered through a 0.22 µm Sterivex filter (Millipore, Inc.) and stored in a -20 °C freezer until processing.

DNA Extraction and Sequencing

Microbiome DNA was extracted using a cell lysis and spin-column filtration method by Macherey Nagel Tissue Kit. The purified DNA was prepared for random shot-gun sequencing

using the standard protocol from Swift 2S Plus Kit (Swift Biosciences). The DNA library was sequenced using a Mi-Seq Illumina sequencer with Mi-Seq v3 Reagent Kit. Samples were bar-coded and the *T. semifasciata* microbiomes were mixed in with a range of microbiome samples and run on several sequencing runs.

Metagenome Annotation

The sequenced metagenomes were processed through PRINSEQ±± software for quality control, removing sequences less than 70 bp, those that had a single N, or a quality score below 25. MID tags were also removed with PRINSEQ±± (Cantu, Sadural, and Edwards 2019; Schmieder and Edwards 2011). The two fasta files were paired using FLASH software (Magoc and Salzberg 2011). After pairing, metagenomes were uploaded into MG-RAST Version 4.0.3, which was used to provide taxonomic and functional gene annotations of the metagenomes using standard cut-offs (Aziz et al. 2008; Meyer et al. 2008). The sequences with the highest bit score were reported. The taxonomic annotation outputs were filtered in MG-RAST to only include those from Bacteria and Archaea domains. Viruses and Eukaryotes were not used in the analysis because they were underrepresented in the metagenome. The functional gene characterization was obtained from the SEED platform (Overbeek et al. 2005), similar to previous analysis (Dinsdale, Edwards, et al. 2008; Dinsdale, Pantos, et al. 2008).

Quantitative evaluation of leopard shark microbiomes

To identify whether the taxonomy of the microbiome remained consistent or varied over time, a Permanova on the relative abundance of each taxon level was conducted across years (Anderson 2017). All data was fourth root transformed to reduce abundance variation among samples. A simper analysis was used to identify the taxa contributing to the dissimilarity between years (Clarke, Somerfield, and Gorley 2008). A principal coordinate analysis was performed on the taxonomic data to visualize the variation in the structure of the microbiomes over the years. β-diversity was identified using a Bray-Curtis analysis with 100 - similarity index, where 0 = high overlap, 100 = no overlap. Last, a PERMDISP analysis compared the distribution of microbial taxa across each year around the year's group centroid (Clarke, Somerfield, and Gorley 2016; Anderson 2006).

We identified a recurrent group of microbes (or core) as those genera that were present on every individual shark, on a presence/absence basis and non-recurrent microbes, as all other genera that were an occasional member of the microbiome. The 19 most abundant recurrent and non-recurrent microbial taxa (abundances calculated within the recurrent/non-recurrent datasets, separately) were compared with a Bray-Curtis similarity analysis to identified groups of genera that had a similar proportional abundance across years. A Pearson correlation was conducted to identify positive or negative correlations between these taxonomic groupings. The output of the Pearson correlation was organized into a heatmap and combined with the Bray-Curtis dendrogram. The analysis identified negative and positive correlations between the microbial groups that live on the shark epidermis.

To identify whether the functional potential of the shark epidermis microbiome remains stable or varies temporally, a PERMANOVA was conducted to compare variation in functional components across years. The SEED provides a hierarchal description including major metabolisms, metabolic pathways, gene clusters and gene functions (Overbeek et al. 2005). The SIMPER analysis identified potential functions that contributed to the dissimilarity between years. A principal coordinate analysis was performed to visualize the variation in the functional potential of the microbiomes over the years and a PERMDISP analysis compared the distribution of functional potential of *T. semifasciata* epidermal microbiome across years. All analyses were performed in PRIMER Version 6.1.15 & PERMANOVA± Version 1.0.5 from PRIMER-E (2012). GraphPad Prism Version 8.3.1 was used to visualize the data.

We identified the functional potential of the 17 most proportionally abundant genera using the Krona plug-in (Ondov, Bergman, and Phillippy 2011) in MG-RAST (Aziz et al. 2008; Meyer et al. 2008). Only 17 genera were use because they occurred in sufficient abundance to explore the functions of the genera. The sequences were further investigated using Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) database's KEGG mapper plug-in on MG-RAST (Kanehisa et al. 2012; Kanehisa et al. 2016), which provided identification of metabolic and biochemical pathways. The proportion of sequences within each genus for each functional group was calculated as the number of sequences within a genus divided by the total number of

sequences within the function across the 17 genera. The proportion of sequences associated with each function in each genus was categorized into one of four quartiles: < 75 %, 50 – 75 %, 25 – 50 %, 1 – 25 %, and 0 % and visualized using a heatmap. The heatmap was used to visualize the connection between the microbes and the sharks over time and identified whether there was switching of specific functional roles between genera in the microbiome.

Results

Temporal Taxonomy of the Epidermal Microbiome

The epidermal microbiomes were constructed from 18 *T. semifasciata*, including seven individuals in 2013, three in 2015 and eight in 2017 caught in La Jolla, California. Metagenomes ranged from 312,135 – 2,347,886 sequences that had 89,080,935 – 605,042,370 base pairs (Table 1). Of those sequences identified as protein coding, 24.01 – 83.74 % were classified as unknown sequences, which could not be matched to database (Table 1). The abundant phyla represented in the *T. semifasciata* epidermal microbiome were Proteobacteria (78.50 ± 2.02 S.E. %), Bacteroidetes (15.83 ± 2.34 %), and Actinobacteria (2.09 ± 0.30 %). Within the Proteobacteria the classes Gammaproteobacteria (38.99 ± 2.28 %) and Alphaproteobacteria (31.32 ± 2.20 %) were evenly represented, with Betaproteobacteria (7.25 ± 0.81 %) and Deltaproteobacteria (0.81 ± 0.05 %) in lower relative abundance (SI Figure 1). Within the Bacteroidetes, the Flavobacteria class made up 12.08 % (± 2.15) of the metagenome and Actinobacteria class accounted for 2.40 % (± 0.30) (SI Figure 2).

Across the 18 leopard sharks, 597 genera made up the epidermal microbiome. Highly represented genera present within the microbiomes included *Pseudomonas* (9.85 ± 1.81 %), *Erythrobacter* (5.88 ± 0.69 %), *Leeuwenhoekiella* (2.52 ± 0.72 %), and *Limnobacter* (1.51 ± 0.42 %) (Figure 1). The *T. semifasciata* microbiomes collected across the three time-points showed high taxonomic similarity between all individuals, with average Bray-Curtis similarity of 82.74 (0 = no overlap, 100 = total overlap) among all pairs of samples. The similarity of the genera on the sharks across time-points suggests a selective process is occurring and that the shark epidermis has a specialized microbiome (Figure 1). The 19 most relative abundant genera (except for *Providencia* 2.29 ± 0.94 %) were recurrent over sampling periods, but the proportional abundance varied throughout the years (Figure 3). The PERMANOVA analysis identified significant differences in the proportional abundances of all taxonomic levels between

years (PERMANOVA: Phylum, Pseudo-$F_{df = 2}$ = 3.696, P(perm) = 0.001; Class, Pseudo-$F_{df = 2}$ = 3.628, P(perm) = 0.001; Order, Pseudo- $F_{df = 2}$ = 3.222, P(perm) < 0.01; Family, Pseudo-$F_{df = 2}$ = 2.762, P(perm) < 0.01; Genus, Pseudo-$F_{df = 2}$ = 2.967, P(perm) = 0.049). The microbial genera of the *T. semifasciata* epidermal microbiomes formed distinct clusters for each year in the principal coordinate analysis, which explained 64.7 % of the variation in the first two axes (Figure 2). A few outliers identified in 2013 and 2017 may be associated with variation in an individual shark's physiology, i.e., such as pregnancy.

The SIMPER analysis identified a similarity coefficient of 83.05 between 2013 & 2015; 80.29 for 2013 & 2017; and 84.64 for 2015 & 2017 (Bray-Curtis similarity index, 100 = similar, 0 = no overlap). Genera of the Gammaproteobacteria class contributed the most to the dissimilarity between the years, followed by genera in the Alphaproteobacteria and Betaproteobacteria, and the Flavobacteria class from the Bacteroidetes phylum (Table 2). The variation in the relative abundance of *Alcanivorax* and *Alteromonas* contributed the most to the dissimilarity across years.

Last, we explored the variance within years of microbial taxonomy with PERMDISP analysis. Each year the microbiome showed a similar amount of variation within the microbiomes at each taxonomic level (PERMDISP: Phylum, $F_{df = 2, 15}$ = 2.120, P(perm) > 0.1; Class, $F_{df = 2, 15}$ = 2.439, P(perm) > 0.1; Order, $F_{df = 2, 15}$ = 1.513, P(perm) > 0.5; Family, $F_{df = 2, 15}$ = 2.019, P(perm) > 0.1; Genus, $F_{df = 2, 15}$ = 2.142, P(perm) > 0.05). The microbiomes from 2013, 2015, and 2017 showed a dispersion of 11.7 (± 1.8), 6.0 (± 0.8), and 10.1 (± 1.3) respectively. In summary, the individual sharks shared many microbial genera suggesting a consistent taxon across the years, with flexibility in the proportional abundance.

Temporally Recurrent Microbes

Microbial genera present in all 18 metagenomes, were categorized as 'recurrent' which included 188 of 597 genera. The stability was considered further by comparing the proportional abundance of sequences within the 188 genera across sampling periods and identified a significant difference (PERMANOVA: Pseudo-$F_{df = 2, 15}$ = 10.462, P(perm) = 0.001). The variability in the recurrent microbial genera, measured by PERMDISP (PERMDISP: $F_{df = 2, 15}$ = 0.029, P(perm) = 0.983) indicated no difference across years with 2013, 2015, and 2017 showing a dispersion of 4.09 (± 0.47), 4.07 (± 0.57), 3.974 (± 0.22), respectively. Collectively the

fluctuations in the dominate genera along with similar variability across time suggest a flexible but regulated recurrent groups of microbes in the *T. semifasciata* microbiome.

Relationship between recurrent and non-recurrent genera

In the clustering analysis, the 19 dominant recurrent microbes formed three groups and the 19 non-recurrent microbes formed five separate groups (Figure 4). The recurrent group A consisted of *Pseudomonas*, *Erythrobacter, Alcanivoriax,* and *Marinobacter*, which showed an even proportional abundance across the five years and with *Erythrobacter and Marinobacter* having higher proportional abundance in 2013. The recurrent group B consisted of seven genera which showed higher proportional abundance in 2017 and included *Leeuwenhoekiella, Altermonas, Gramella, Zunongwangia, Pseudoaltermonas, Flabacterium,* and *Maribacter*. The recurrent group C consisted of six genera with lower proportional abundance, including *Burkolderia, Novoshingobium and Sphingopyxis,* which showed even distribution across the years, *Rugeria*, which showed proportional higher abundance in 2013 and 2017 and *Sphingobium* and *Sphingomonas*, which showed higher proportional abundance in 2015. The non-recurrent (top 19 most relative abundant genera not occurring across all sampling points) microbial group A consisted of three genera with lower proportional abundance in 2017, including *Propionibacterium, Pirellula*, and *Plesiocystis*. The non-recurrent microbial group B consisted of two genera that showed low but even proportional abundance across all years and included *Aromatoleum* and *Chromobacterium*. The non-recurrent microbial group C only consisted of *Dokdonia*, which displayed higher proportional abundance in 2017. The non-recurrent group D included *Acidiphillium, Azorhizobium, Gluconobacter,* and *Sphingobacterium* and the first two genera displayed low even proportional abundances throughout all years *Gluconobacter* and *Sphingobium* showed higher proportional abundance in 2017. The non-recurrent microbial group E, including *Leadbetterella, Klebsiella, Neisseria, Oligotropha, Thauara,* and *Frankia. Leadbetterella* displayed its highest proportional abundance in 2015. *Klebsiella, Neisseria,* and *Thauara* showed even proportional abundance across all years. *Oligotropha* and *Frankia* displayed lower proportional abundances at all years (Figure 4). There also occurred a clustering group termed ‘mixed’ because genera included those which were classified as both recurrent and non-recurrent including Endoritia, Limnobacter, Xylella, and Providencia genera.

We then investigated the correlative relationship of recurrent and non-recurrent groups (Figure 4). The heatmap showed that *Erythrobacter* and *Marinobacter* (recurrent group A) were negatively correlated with all microbes in recurrent group B. Whereas *Pseudomonas* and *Alcanivorax* (recurrent group A) showed positive correlations with recurrent group B. Recurrent group A, however, was positivity correlated with non-recurrent group A and B. In contrast, recurrent group B was negatively correlated with non-recurrent group A and B but strongly associated with non-recurrent group C, *Dokdonia*. Recurrent group C was positively correlated with non-recurrent groups A, B, D, E, and the mixed group (Figure 4). These associations between taxa will be investigated further in the functional analysis to identify metabolisms that may drive the microbiome composition.

Functional potential of the shark epidermis microbiome

Annotated microbial sequences were categorized into 26 major metabolic groups, 153 metabolic pathways, 949 functional gene clusters, and 5,358 functional genes. The gene function profiles were highly stable across the three time-points as evidenced by the high functional similarity between individual sharks (mean 97.65, Bray-Curtis similarity index). There was not a significant difference between time-points in the functional potential of the microbiome (PERMANOVA: major metabolic groups, Psuedo-$F_{df = 2, 15} = 1.8$, P(perm) > 0.05; metabolic pathways, Psuedo-$F_{df = 2, 15} = 0.525$, P(perm) > 0.5; functional gene clusters, Pseudo-$F_{df = 2, 15} = 1.182$, P(perm) > 0.1). The similarity of the functional potential was shown by the single cluster on the principal coordinate analysis that explained 64.4 % of the variation in the first two axes (Figure 5).

The major metabolic groups included carbohydrates (mean 10.96 ± 0.32 %); amino acids & derivatives (9.17 ± 0.19 %); protein metabolism (7.88 ± 0.11 %); and fatty acids, lipids, & isoprenoids (2.61 ± 0.04 %) (SI Figure 3). In nitrogen metabolism (1.23 ± 0.11 %), there were 11 functional gene clusters in the microbiome including ammonia assimilation (0.51 ± 0.01 %), nitrate and nitrite ammonification (0.38 ± 0.001 %), and denitrification (0.10 ± 0.02 %), but few sequences were associated with nitrogen fixing genes (0.02 ± 0.007 %) suggesting high levels of organic nitrogen that are present on the shark. Urea decomposition (0.27 ± 0.02 %) was present and there were a few sequences associated with trimethylamine N-oxide (TMAO) reductase (0.004 ± 0.001 %). The functional gene clusters that perform fatty acid biosynthesis and

metabolism are fatty acid biosynthesis FASII (0.72 ± 0.02 %), fatty acid degradation regulons (0.22 ± 0.02 %), and fatty acid metabolism (0.13 ± 0.01 %). The functional gene clusters that complete protein biosynthesis and degradation are ATP dependent proteolysis in bacteria (0.37 ± 0.01 %), bacterial proteasome (0.30 ± 0.02 %), protein degradation (0.20 ± 0.01 %), nucleolar protein complex (1.01 ± 0.05 %), aminopeptidase (0.18 ± 0.01 %), metallocarboxypeptidase (0.04 ± 0.006 %), and bacterial ribosome LSU (0.38 ± 0.02 %). A high proportional abundance of flagellar motility (1.27 ± 0.06 %) and lower proportional abundance of bacterial chemotaxis (0.36 ± 0.02 %) is consistent with low biofilm formation predicted on the shark epidermis. There was a high proportional abundance of functions associated with resistance to antibiotics and toxic compounds (5.08 ± 0.13 %) and cobalt-zinc-cadmium resistance had a mean of 1.58 ± 0.12 %, multidrug resistance efflux pumps (0.85 ± 0.05 %), copper homeostasis (0.69 ± 0.05 %), and the production of exopolysaccharide biosynthesis (EPS) (0.058 ± 0.008 %) and Rhamnolipids produced by *Pseudomonas* (0.005 ± 0.002 %) that are used in heavy metal detoxification, consistent with bioaccumulation of heavy metals and toxins by the large-bodied sharks.

Microbial gene function across genera

The 17 dominant recurrent genera were used to investigated whether functional genes were equally dispersed across the genera or confined to a small subset of microbes (Figure 6). *Pseudomonas*, had the highest proportion of sequences annotated to all metabolisms, including those involved in nitrate and nitrite ammonification and denitrification, and in association with *Alcanivorax*, also conducted ammonia assimilation. These two genera were positively correlated (Figure 6) with most other microbes, suggesting these were key functions in microbiome growth. In comparison, *Marinobacter* also specialized in these two functions plus urea decomposition and was negatively correlated *Pseudomonas* and *Alcanivorax. Ruegeria* and *Novosphingobium* were the only other genera that had a high proportion abundance of genes associated with urea decomposition, which may have caused the negatively correlated with recurrent group B. The genera within recurrent group B had a high proportion of genes associated with carbon metabolism, with *Gramella* having a high proportion of genes within TCA cycle, Glycolysis and Serine cycle. Maribacter has a high proportion of genes in Glycolysis, Denitrification, and fatty acid biosynthesis. Many genera, including *Pseudomonas, Alcanivorax, Erythrobacter, Leeuwenhoekiella, Altermonas, Gramella* and *Flavobacterium,* had many sequences associated

with protein biosynthesis and degradation, and fatty acid biosynthesis, which are associated with the shark ketosis dominated metabolism. Genera within recurrent group C, including *Ruegeria*, *Sphingopyxis* and *Novosphingobium* had a high proportion of genes associated with Denitrification and fatty acid biosynthesis. All 17 genera, except 1, had sequences that matched the copper, cobalt and cambium resistance and copper homoeostasis genes. The abundance of these functions across most genera in the metagenomes suggests the important heavy metal tolerance or manipulation for microbes living on the surface of sharks.

Discussion

The *T. semifasciata* epidermal microbiome was consistent between individual sharks with many genera shared across the three time-points spanning 5 years, one of the longest studies of marine vertebrate microbiomes to date. A recurrent set of 188 microbial genera were present on all individuals. However, there was flexibility in the proportional makeup of the microbiome across years, but the microbiome variability was consistent across years, suggesting similar ecological dynamics are occurring from year to year, despite some turnover in microbial genera. Regardless of the proportional abundance of each microbial genera, the functional potential was constant, suggesting that the epidermal microbes were responding to the unique environment of the *T. semifasciata* epidermis, including the dermal denticles that reduce biofilm build-up and potentially elements secreted via the minimal mucus associated with shark's metabolism.

Leopard shark microbiome exhibits specificity and flexibility

The *T. semifasciata* epidermal microbiome has a high relative abundance of *Pseudomonas, Erythrobacter, Alcanivorax,* and *Marinobacter*; whereas the thresher shark (*Alopias vulpinus*) epidermal microbiome had a high relative abundance of *Pseudoalteromonas, Erythrobacter, Idiomarina, and Limnobacter* (Doane et al. 2017); and the *Carcharhinus melanopterus* epidermal microbiome a high relative abundance of *Psychrobacter, Pseudoalteromonas, Rhodobacter*, and *Alteromonas* (Pogoreutz et al. 2019). While some genera were shared across shark species, the proportional abundance differed, suggesting that each elasmobranch microbiome has distinct characteristics which select for differing microbiome members.

455

456 The highly shared microbiome across individual sharks within a timepoint and flexible
457 communities across timepoints coupled with evidence that species microbiomes are distinct may
458 be caused by the dermal denticles (Chien et al. 2020; Dillon, Norris, and O'Dea 2017) and
459 metabolites secreted in the skin mucus as a result of the sharks metabolism. Dermal denticles
460 alter the hydrodynamic properties of water close to the epidermis, reducing drag on the shark
461 (Sullivan and Regan 2011a) and micro-organism growth (Wen et al. 2015; Zhang et al. 2011). A
462 study that tested the recruitment of two lab microbial species found that only one species was
463 able to successfully recruit, and biofilms did not develop (Chien et al. 2020; Kim et al. 2012).
464 Biofilm development is dependent on quorum sensing, which is interrupted on rough surfaces
465 (Kim et al. 2012). We showed that specific microbial taxa recruit to the epidermis of *T.*
466 *semifasciata* and the proportion of motility genes are relatively high compared to the chemotaxis
467 genes which are proportionally low in abundance in the leopard shark microbiomes. These
468 features are consistent across the microbiome of four shark species (Doane et al. 2017; Doane et
469 al. 2020).
470

471 Modelling of microbial community dynamics of surfaces with structural complexity, like the
472 conditions on a sharkskin, potentially suggests there is lower interspecific competition, which
473 results in a chaotic spatial distribution pattern of microbial species (Lowery and Ursell 2019).
474 The *T. semifasciata* epidermal microbiome supports the modelling where 188 genera coexisted
475 on all sharks across the three years, with flexibility in the relative abundance of those genera,
476 suggesting that the epidermis may provide multiple niches for microbial taxa to survive. An
477 emergent feature of the modelling is that competing genera do not go extinct (Lowery and Ursell
478 2019), and we show that many microbial species co-occurred on the sharkskin across the three
479 time-points. The co-occurrence or positive correlations within the microbiome suggest niche
480 partitioning and sharing of resources with several genera exhibiting abundance relationships
481 (Figure 4). For example, *Pseudomonas* and *Alcanivorax* showed positive correlations with
482 recurrent group B members, including *Leeuwenhoekiella, Altermonas, Pseudoaltermonas,*
483 *Flabacterium,* and *Maribacter*, suggesting that these two genera could share
484 resources. *Erythrobacter* and *Marinobacter* were negatively correlated with all microbes in
485 recurrent group B, suggesting strong competition; this group was however positively correlated

with non-recurrent group A and B, suggesting transient microbes add to the microbiome's growth at different years.

Microbiome functional groups

We then investigated the gene functions present in the groups of microbial genera that may explain the positive or negative relationships between genera in Figure 4. In addition, because host metabolism and microbial functions are linked (Lynch and Hsiao 2019), we identified functional genes in each genus that may be linked to the host metabolism and classify three broad functional groups, generalists (recurrent group A), opportunists (recurrent group B) and specialists (recurrent group C) based on the presence of potential genes in these microbial genera. The group A recurrent microbes are typical marine species that have highly flexible genomes and growth strategies, including heterotrophic and photo-heterotrophic growth. These organisms can utilize many of the nutrients that may be present on the shark epidermis or excreted in the mucus, including proteins, fatty acids and disulphides (Meyer and Seegers 2012). *Pseudomonas*, for example, had the highest relative abundance and are capable of mixotrophy and simultaneous use of sulphur, nitrogen, and carbon from the surrounds (Guo, Chen, and Lee 2019) and sequences similar to these genes were highly represented in the *Pseudomonas* in leopard shark microbiomes. *Pseudomonas* were positively correlation with many microbes (Figure 4), which may be enhanced by traits, such as phosphate solubilization, and ammonia production (Yang et al. 2019; Goswami et al. 2013). Several, *Pseudomonas* species poses urease activity (Goswami et al. 2015) and tolerate, transition metals via the production of extracellular polysaccharides (EPS), such as Rhamnolipids, that form complexes with heavy metals (Meliani 2015).

Alcanivorax species, a highly abundant recurrent group A microbe, reduced alkane hydrocarbons, and possessed many enzymes for β-oxidation of fatty acids, the glyoxylate bypass, and the gluconeogenesis. These gene functions carried by *Alcanivorax* suggest they break down the complex hydrocarbons and fatty acid rich metabolites and may facilitate other member of the microbiome by providing simpler metabolic products that can be utilized. They also include enzymes for synthesis of riboflavin and unsaturated fatty acids and cardiolipin (Sabirova et al. 2020). Cardiolipin is a diphosphatidylglycerol lipid (Schlame, Brody, and Hostetler 1993) and is

more highly saturated in sharks than other vertebrates (Shadwick, Farrell, and Brauner 2015) and sequences similar to Cardiolipin were identified in the metagenomes.

Erythrobacter species, a highly abundance group A microbe that was negatively correlated with many other microbes are mostly aerobic anoxygenic phototrophic bacteria, containing bacteriochlorophyll a, but lack the genes of autotrophic CO_2 fixation pathway, thus photoheterotrophic metabolism requiring a supply of organic substrates. Several *Erythrobacter* species have genes encoding enzymes for glycolysis and the tricarboxylic acid cycle but lack genes for nitrogenase or nitrate reductase thus are reliant on other forms of organic nitrogen (Koblizek et al. 2003), which matches the lower proportion of sequences identified in the *Erythrobacter* in the *T. semifasciata* microbiome. *Erythrobacter* carry heavy metal resistance genes, including resistance to lead, cadmium, zinc, mercury, nickel, cobalt, and arsenicals (Zheng et al. 2016) and these genes were highly present in the metagenomic profiles. The behaviour of the leopard sharks, being in shallow water with higher light conditions may have provided the *Erythrobacter* with a competitive advantage over other microbial groups.

The last group A recurrent microbe was *Marinobacter* and these organisms are opportunistic generalists that switch rapidly between lithoheterotrophy to heterotrophy, in both anaerobic and aerobic conditions, in response to nutrient pulses. They have fast growth, outgrowing other species in culture (Handley and Lloyd 2013). *Marinabacter* are able to respire inorganic compounds that are usually found in metal rich environments (Handley and Lloyd 2013), and we show they have many genes associated with metal resistance and transport. *Marinobacter* use many hydrocarbons as a sole energy source, and produce large quantities of bioemulsifier, which aid in bacterial adhesion to hydrophobic surfaces.

The recurrent group B microbes, the opportunists, were negatively correlated with most other groups and had a high relative abundance of genes associated with carbon metabolism. *Gramella* species degrades high molecular weight organic matter and encoding hydrolytic enzymes predicting a preference for polymeric carbon sources and have range of gliding motility that provide for surface adhesion (Bauer et al. 2006), features that enable the organism to live on the surface of elasmobranchs. However, these microbes had fewer genes in ammonia assimilation, promoting their coexistence with the more abundant species. *Leeuwenhoekiella*, a marine Flavobacteria, has guiding motility, heterotrophic growth, is pigmented and often found in

associated with photosynthetic microbes and converts nitroalkanes into nitrite (Tahon et al. 2020). *Alteromonas* are non-phototrophic, heterotrophic, with flexible genome for taking advantage of influx of nutrients, particularly polymers, and some degrade polycyclic aromatic hydrocarbons (Math et al. 2012). *Alteromonas* have high flexibility in the metal resistant genes and efflux pumps and show strain differences in the copper-zinc-colbalt gene (czc) cassettes by successive insertions at a tRNA gene, with the numbers of czc cassettes varying from zero to three (Lopez-Perez et al. 2012) and these highly abundant in this genus in the *T. semifasciata* microbiome. *Pseudoalteromonas* and *Alteromonas* utilized extracellular hydrolysis as the major decomposition pathway of peptides and released fragments of amino acids into the surrounding environment (Liu and Liu 2020) and may have enable them to outcompete *Marinobacter* which is unable to conduct extracellular hydrolysis processes. However, these free amino acids may be used by other microbes, such as the non-recurrent group C and D microbes.

The group C recurrent microbes, the specialists, had less relative abundance overall but showed high relative abundance of a few metabolic pathways (Figure 5). *Ruegeria* had a high proportion of sequences associated with urea metabolism, compared with its relatively low proportional abundance. *Ruegeria* are able to use Trimethylamine (TMA) and trimethylamine N-oxide (TMAO) as an energy source to product intracellular ATP (Lidbury, Murrell, and Chen 2015). TMAO and TMA are used by elasmobranchs for osmoregulation.

Sphingopyxis also had low relative abundance, utilizing carbon and nitrogen compounds as substrates for growth but are generally slow growing (Moran et al. 2007). Serine, a pathway that was overrepresented in *Sphingopyxis*, can serve as a nitrogen source for the growth of some species, but not as a carbon source (Williams et al. 2009). Ammonia and nitrate are used as nitrogen sources, but there is a high energetic cost associated with nitrate utilization (Verma et al. 2020). *Sphingomonas* are closely related to *Sphingoyxis* and both produces exopolysaccharides (EPS) (Chang et al. 2021). The production of these compounds may provide biofilm development on the shark skin. Therefore, each recurrent microbial group was providing a different set of functional genes that enhanced coexistence and reflected the products that may be associated with shark metabolism and excreted via the mucus.

Gene functions linked to host metabolic processes

576 The stability of the functional potential of the epidermal microbiome suggests the elasmobranch
577 physiology and dermal denticle topography are strong drivers of microbiome function, and we
578 have developed a model to link the microbiome function with shark metabolism (Figure 7).
579 Elasmobranchs rely on protein as the primary food source and use ketogenic pathways and
580 produce high ketone bodies and hydrocarbons (Speers-Roesch and Treberg 2010). The functions
581 that were present in metagenomes suggests that the microbes were responding to the presence of
582 these compounds in the minimal mucus that covers the sharks. For example, *Alxavorax* and
583 *Marinobacter* carried the genes to degrade hydrocarbons. There are few descriptions of the
584 compounds within the mucus of Elasmobranchs, and these included high levels of proteins
585 (Tsutsui et al., 2009) with moderate to high disulphide concentrations (Meyer et al., 2007a). A
586 recent transcriptome analysis of the integument of cookie cutter sharks identified
587 Glyceraldehyde-3-phosphate dehydrogenase and Fructose-bisphosphate aldolase A transcripts,
588 both play key roles in the glycolysis and gluconeogenesis (Delroisse et al. 2021) suggesting these
589 products are being produced in the skin and may be available to the microbial community.
590 Protein metabolism and nitrate and nitrite ammonification, urea decomposition with genes such
591 as trimethylamine N-oxide (TMAO) reductase were significant functions in the microbiomes.
592 The low levels of nitrogen fixation in the microbiome and the high proportional abundance of
593 nitrogen metabolism and urea decomposition and the presences of within the microbiome
594 suggest the elasmobranch is a nitrogen-rich environment (Hazon et al. 2003; Dowd et al. 2010).
595 Proteasomes, metallocarboxypeptidase, aminopeptidase genes, protein degradation and
596 biosynthesis functional genes suggest a propensity of the microbes for protein degradation. There
597 was no evidence of dentine, hydrolysate, or collagen degradation in the microbiome, suggesting
598 that the microbes are not breaking down the dermal denticles, which signifies a lack of negative
599 impact on the sharks. Therefore, a commensal connection between *T. semifasciata* and the
600 epidermis microbiome is established through the passive resource subsidization of these
601 microbiome functions.
602
603 Cobalt-zinc-cadmium resistance and ton/to transport genes are highly prevalent functional genes
604 shared across epidermis microbiomes of *T. semifasciata* (this study), *A. vulpinus* (Doane et al.
605 2017), *Rhincodon typus* and *Urobatis halleri* (Doane et al. 2020). Elasmobranchs bioaccumulate
606 heavy metals and shunt these compounds to the epidermis (Escobar-Sanchez, Galvan-Magana,

and Rosiles-Martinez 2010; Maz-Courrau et al. 2012), and the epidermal microbiomes are responding to the presence of these compounds. The prevalence of heavy metal resistance genes may provide a biomarker of elasmobranchs health and identify those exposed to anthropogenic sources of heavy metals in the environment providing an early warning sign of declining health.

Conclusions

The highly structured epidermis of elasmobranchs, in this case, *T. semifasciata*, promotes a diverse microbiome that has a flexible taxonomic makeup where many genera coexist. The microbial community was maintained by functional redundancy driven by the taxonomic flexibility and various microbes "flip-flopping" in abundance with one another, which kept the microbial niches filled across the years. The high relative abundance of genes involved in nitrification and denitrification, urea decomposition, and heavy metal resistance genes in the epidermis microbiome across all *T. semifasciata* individuals suggests a connection with the host metabolism potentially through passive subsidization of organic nitrogen, proteins, hydrocarbons, and heavy metals.

Abbreviations

Not applicable

Ethical Approval and Consent to participate

Animal handling and ethics were reviewed at San Diego State University through IACUC under permit APF #14-05-011D, APF #17-11-010D, APF # 18-05-007D. Sampling was conducting under state permit SCP #12847 and SCP #9893 from the California Department of Fish and Wildlife.

Consent for publication

Not applicable

Availability of data and materials

All data will be publicly released upon publication.

Competing interests

The authors declare no conflict of interest.

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Author's contribution

MD collected all field samples, constructed the ideas and help write the paper, CJ analyzed the data and wrote the manuscript, EK, AT, AG, SJ, captured and prepared samples and provide feedback on the manuscript, MMM and LL provided sequencing and analysis, AN facilitated the catching of leopard sharks, RD provided the illustration, ED conceptualized the idea, analyzed, wrote and lead the project.

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Tables and Figures

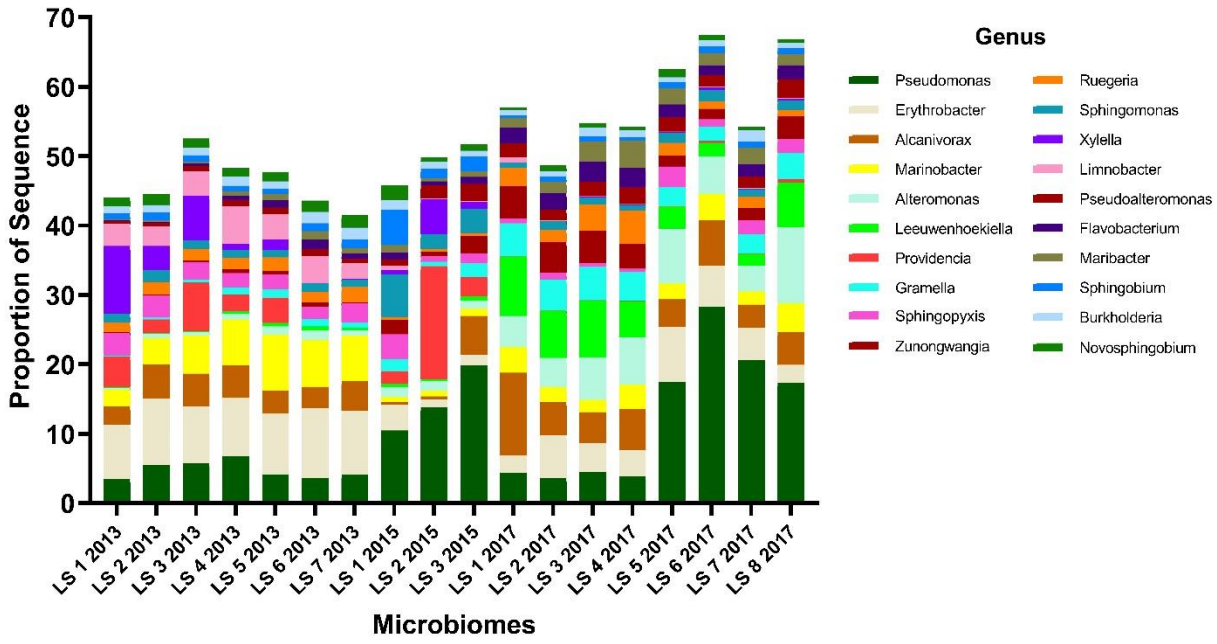

Figure 1. The relative proportions of the most abundant microbial genera represented in the *Triakis semifasciata* microbiome across four years.

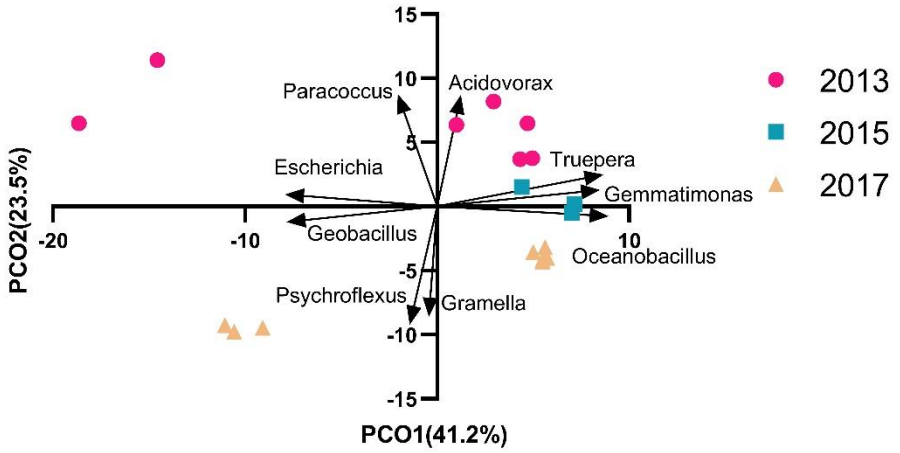

Figure 2 The microbial genera of the *Triakis semifasciata* epidermal microbiome clustered by year, with a few outliers on the principal component analysis conducted on proportional sequence distribution.

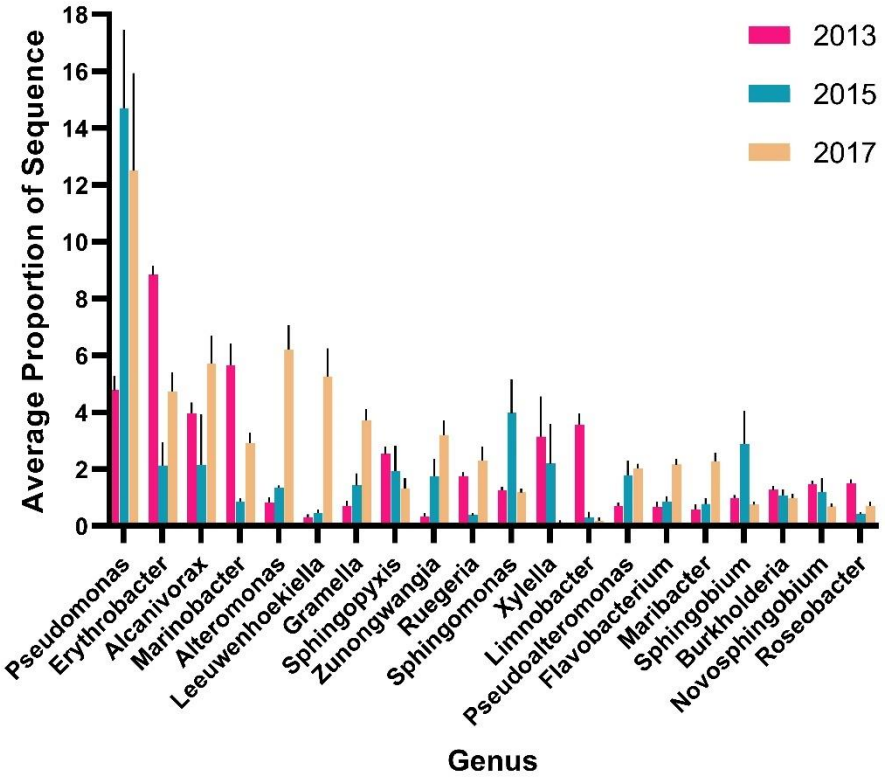

Figure 3. The mean relative abundance (± S.E.) of abundant recurrent genera per year on the *Triakis semifasciata* epidermal microbiome, showing flexibility with no abundant genera becoming extinct over the four years.

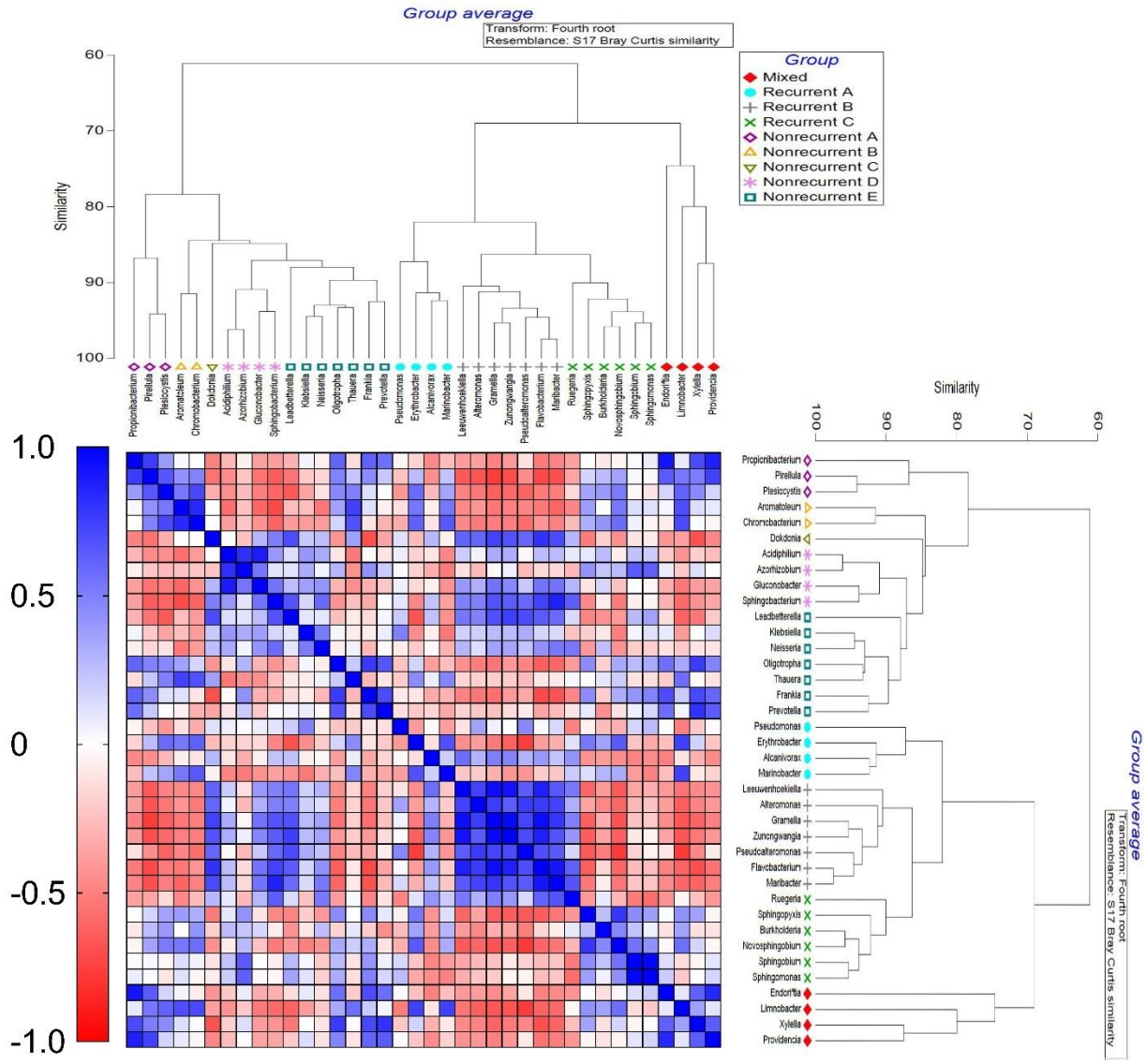

Figure 4. Positive and negative correlations occurred between most abundant 19 recurrent and non-recurrent microbes in the *Triakis semifasciata* epidermal microbiome. Bray Curtis similarity was used to cluster the genera with similar proportional abundance across years and the relationship with other genera was compared using a Pearson's correlation, displayed as a heat map where red is a negative correlation and blue is a positive correlation.

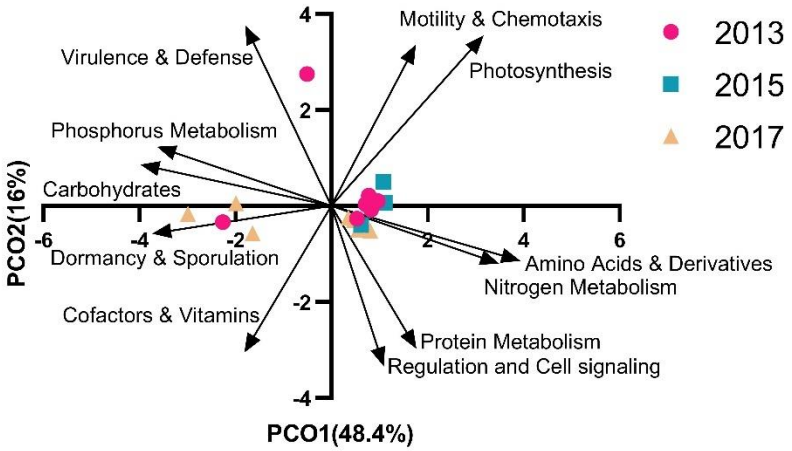

Figure 5. The functional potential of the *Triakis semifasciata* epidermal microbiome was stable across the four-year period, shown using a principal component analysis.

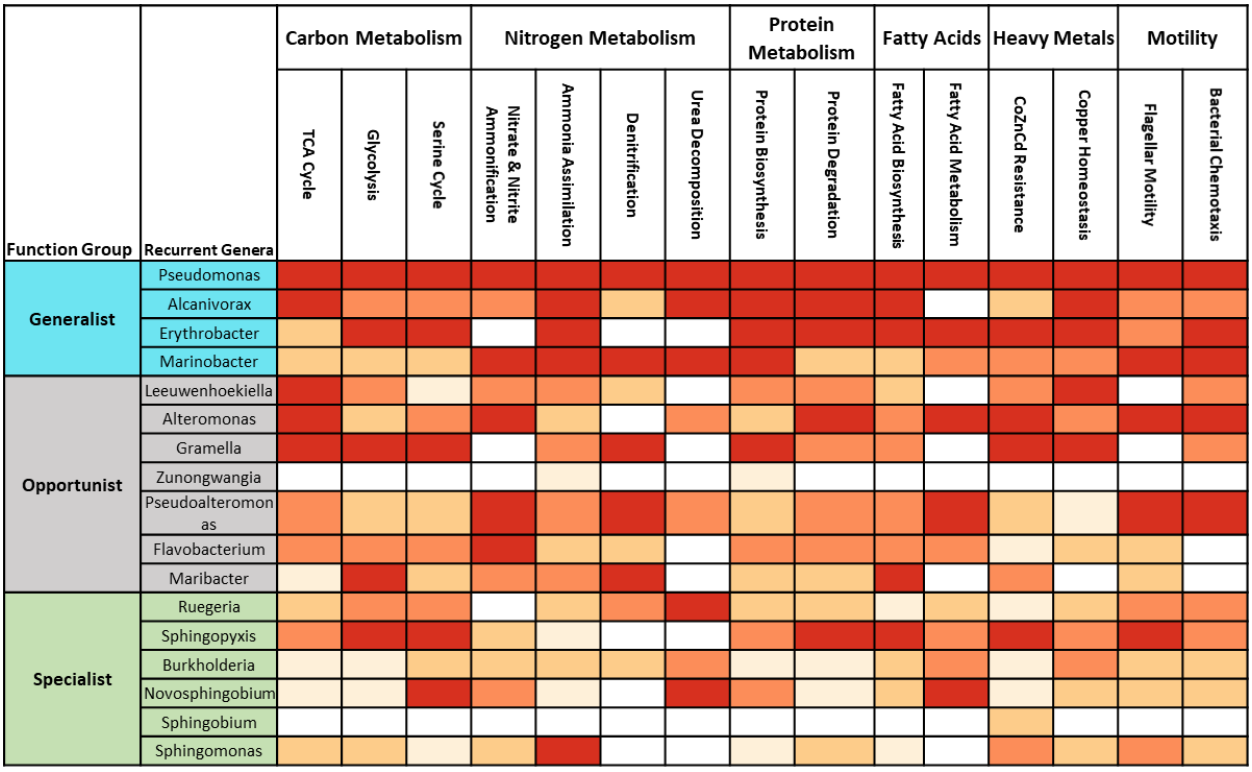

Figure 6. Heat map of the mean proportion of genes associated with each metabolism in the 17 most abundant recurrent taxon in group A (aqua), group B(grey) and group c (green), shows genes were mostly shared across taxon, but some specialization occurred. The proportion of sequences were divided into quartiles, where darker colors represent higher percentile (>75– dark red, 50-75 orange/red, 25-50 - orange, and 0-25 - pale orange). No color represented no sequences for that metabolism held by that genus.

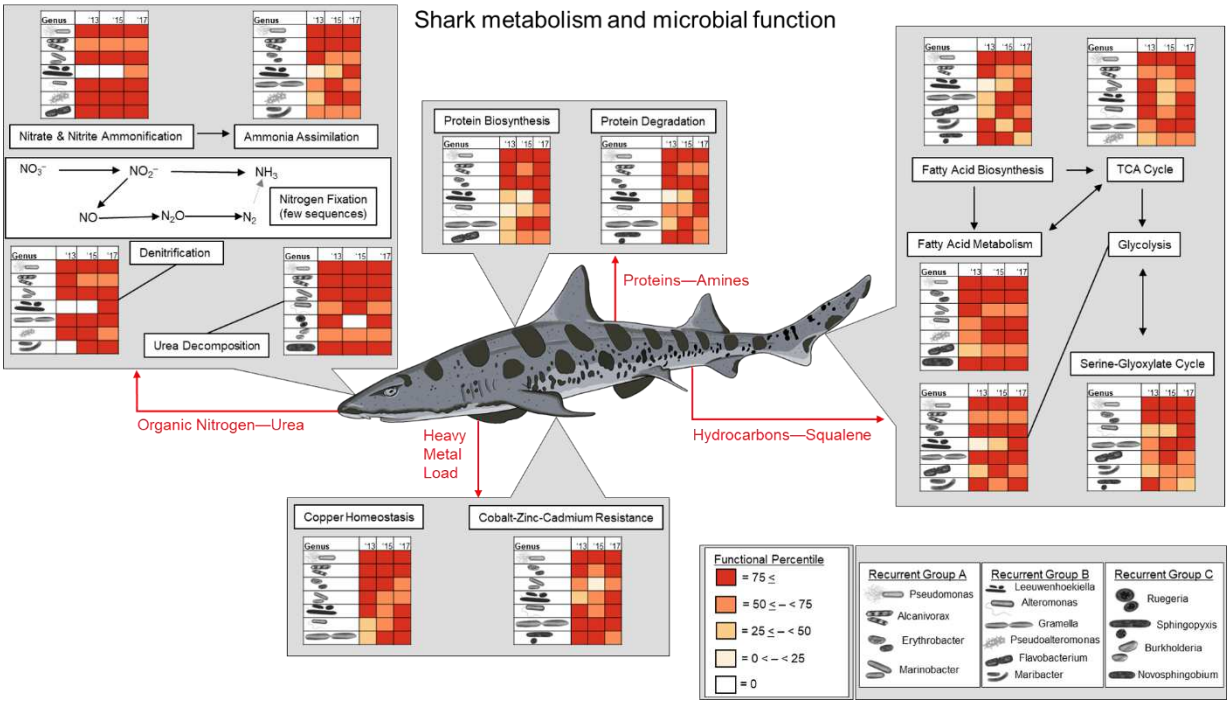

Figure 7. The links between the functional potential of the *Triakis semifasciata* epidermal microbiome and the host metabolism. The proportion of each gene in each taxon were identified and classed into quartiles. Most taxa had most functions suggesting the microbiome displays weak competition and is responding to the metabolic processes of the host.

742 Table 1. The *Triakis semifasciata* metagenome statistics, including collection date, total sequences and
743 proportion of the metagenome that was annotated by the database.

Metagenome Name	Date	Location	SRA#	Number of sequences	Annotated proteins (%)
LS 1 2013	2013	La Jolla		1188249	20.69
LS 2 2013	2013	La Jolla		1060680	24.36
LS 3 2013	2013	La Jolla		1024296	20.16
LS 4 2013	2013	La Jolla		1154296	21.33
LS 5 2013	2013	La Jolla		2347886	19.79
LS 6 2013	2013	La Jolla		3346424	18.84
LS 7 2013	2013	La Jolla		2330953	20.36
LS 1 2015	2015	La Jolla		1143301	22.68
LS 2 2015	2015	La Jolla		2012275	15.87
LS 3 2015	2015	La Jolla		1635251	20.4
LS 1 2017	2017	La Jolla		701418	75.63
LS 2 2017	2017	La Jolla		898767	74.86
LS 3 2017	2017	La Jolla		636435	46.71
LS 4 2017	2017	La Jolla		324102	68.57
LS 5 2017	2017	La Jolla		312135	61.76
LS 6 2017	2017	La Jolla		693318	43.43
Ls 7 2017	2017	La Jolla		613946	62.52
LS 8 2017	2017	La Jolla		1189453	43.69

744

745

746 Table 2. The genera that contributed to the flexibility in the *Triakis semifasciata* epidermal
747 microbiome across four years, calculated via a Simper analysis, with dissimilarity coefficient of
748 16.95 between 2013 & 2015; 19.71 for 2013 & 2017; and 15.32 for 2015 & 2017
749

Years compared	Phyla	Class	Genus	Percent Contribution
2013 vs 2015	Proteobacteria	Gammaproteobateria	*Providencia*	0.9
			Endorifia	0.86
			Marinobacter	0.72
			Pseudomonas	0.61
			Xylella	0.58
			Alcanivorax	0.58
		Betaproteobacteria	*Liminobacter*	0.87
		Alphaproteobacteria	*Erythrobacter*	0.7
			Hyphomonas	0.56
			Oceanibulbus	0.66
2013 vs 2017	Bacteroidetes	Flavobacteria	*Leeuwenkoekiella*	0.9
			Zuongwangia	0.7
			Gramella	0.58
	Proteobacteria	Betaproteobacteria	*Limnobacter*	0.89
		Gammaproteobateria	*Providencia*	0.89
			Alteromonas	0.73
			Xylella	0.67
			Endorifia	0.54
		Alphaproteobacteria	*Hyphomonas*	0.48
		Planctomycetia	*Planctomyces*	0.47
2015 vs 2017	Proteobacteria	Gammaproteobateria	*Providencia*	1.41
			Endorifia	1.11
			Psychrobacter	0.91
			Xylella	0.81
			Alcanivorax	0.75
			Altermonas	0.69
			Pseudomonas	0.52
		Alphaproteobacteria	*Ruegeria*	0.59
		Betaproteobacteria	*Achromobacter*	0.56
	Bacteroidetes	Flavobacteria	*Leeuwenkoekiella*	0.95

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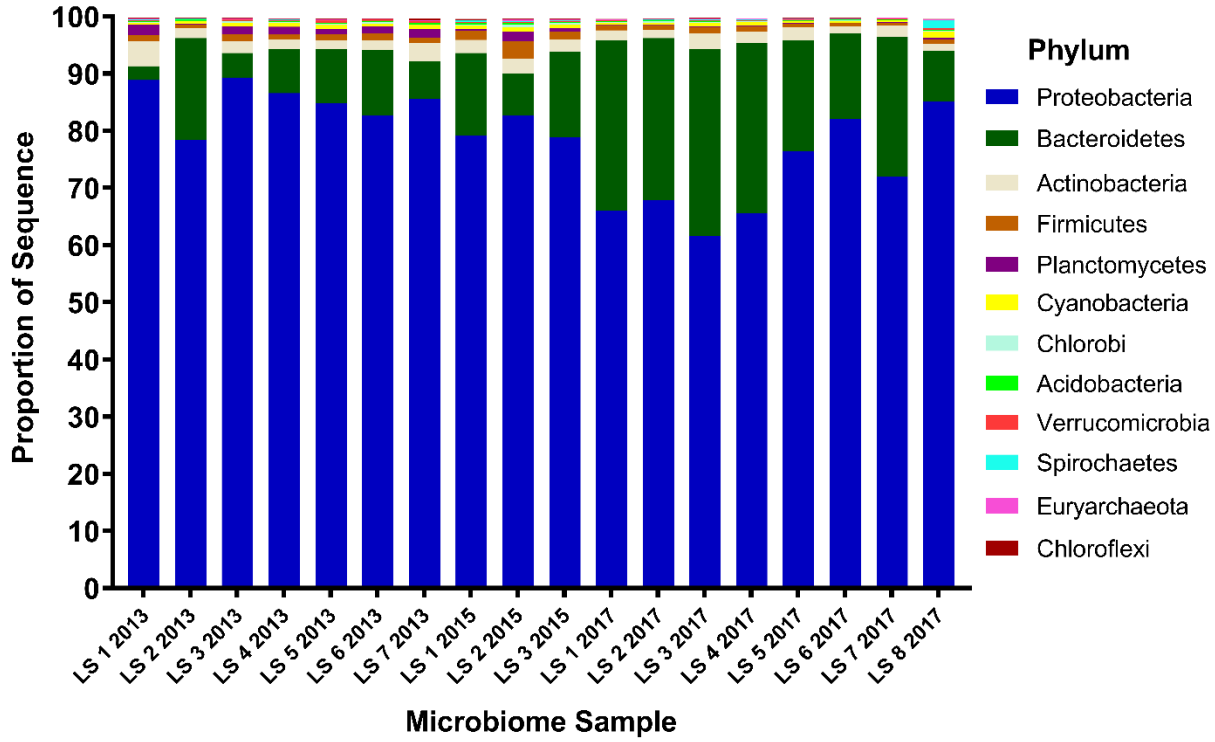
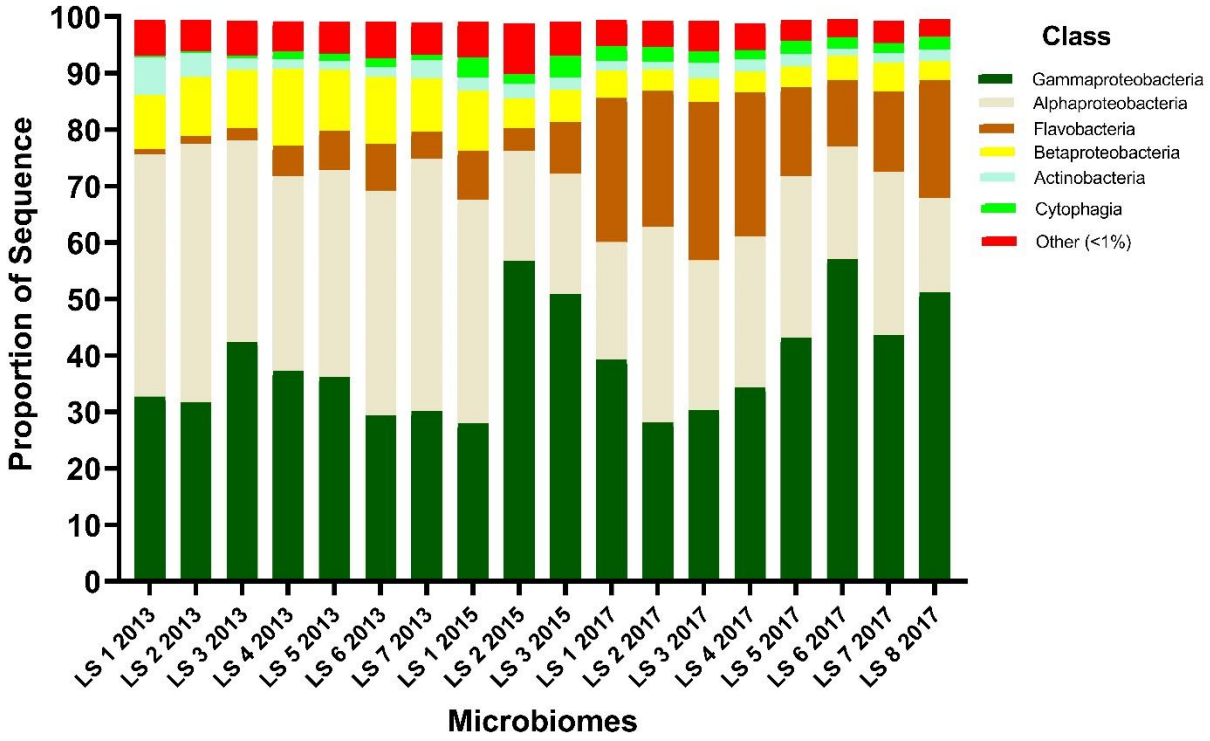
Supplementary material

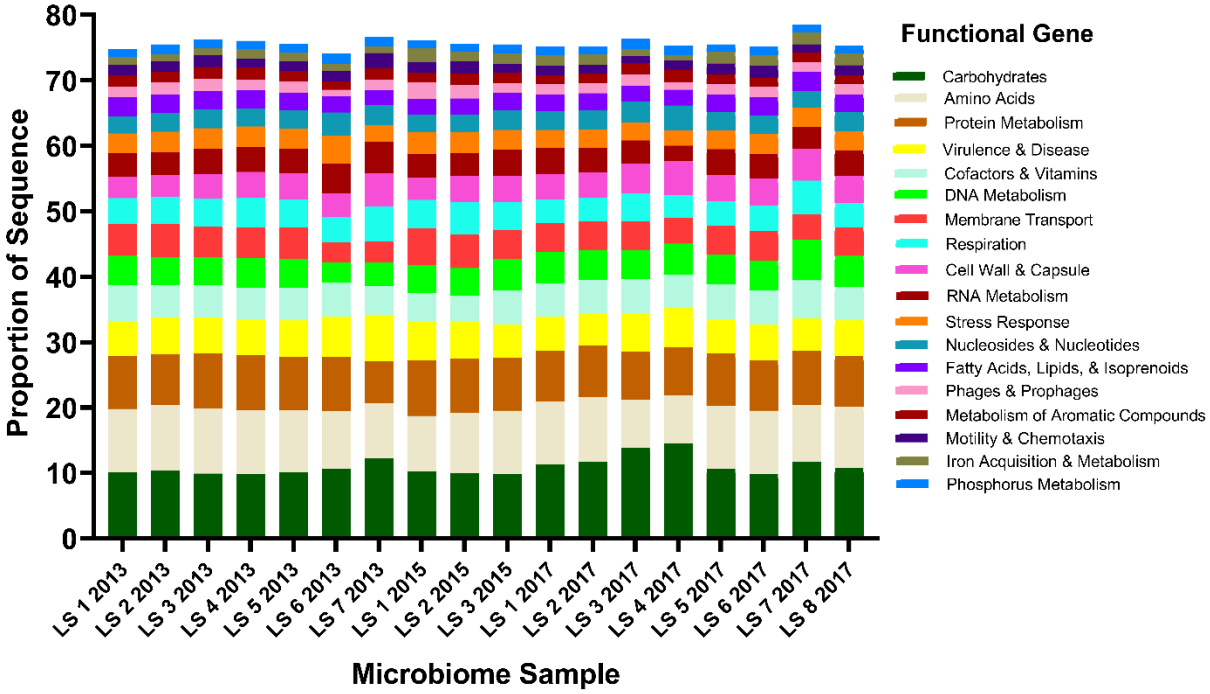


Supplementary Figure 1. The phylum profile of the *Triakis semifasciata* epidermal microbiome had stable across four years.



Supplementary Figure 2.

The relative distribution of microbial classes in the *Triakis semifasciata* microbiome were similar over a four-years.



Supplementary Figure 3. The major metabolisms identifying within the *Triakis semifasciata* microbiomes.

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